

Development and Use of a Selective Medium for Isolation of *Leuconostoc* spp. from Vegetables and Dairy Products†

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A selective medium (LUSM medium) for the isolation of *Leuconostoc* spp. was developed. This medium contained 1.0% glucose, 1.0% Bacto Peptone (Difco), 0.5% yeast extract (BBL), 0.5% meat extract (Difco), 0.25% gelatin (Difco), 0.5% calcium lactate, 0.05% sorbic acid, 75 ppm of sodium azide (Sigma), 0.25% sodium acetate, 0.1% (vol/vol) Tween 80, 15% tomato juice, 30 µg of vancomycin (Sigma) per ml, 0.20 µg of tetracycline (Serva) per ml, 0.5 mg of cysteine hydrochloride per ml, and 1.5% agar (Difco). LUSM medium was used successfully for isolation and enumeration of *Leuconostoc* spp. in dairy products and vegetables. Of 116 colony isolates obtained from fresh raw milk, curdled milk, or various vegetables, 115 were identified as members of the genus *Leuconostoc*. A total of 89 of these isolates were identified to species; 13.5% of the isolates were *Leuconostoc cremoris*, 7.9% were *Leuconostoc mesenteroides* subsp. *mesenteroides*, 11.2% were *Leuconostoc mesenteroides* subsp. *dextranicum*, 16.9% were *Leuconostoc mesenteroides* subsp. *paramesenteroides*, 10.1% were *Leuconostoc lactis*, and 40.4% were *Leuconostoc oenos*. When we compared the counts obtained for two *Leuconostoc* strains, *Leuconostoc dextranicum* 181 and *L. cremoris* JLL8, on MRS agar and LUSM medium, we found no significant difference between the values obtained on the two media.

Leuconostocs are heterofermentative lactic acid bacteria that occur naturally in milk, grass, herbage, grapes, and many vegetables (23). Members of this group are used in dairy fermentations to produce aroma compounds (3). Attempts have been made to develop media for the isolation and enumeration of these organisms, and both selective and differential media have been described; however, no medium has proven to be satisfactory.

Comprehensive reviews of *Leuconostoc* differential and selective media have been published by Garvie (9), Teuber and Geis (23), and Cogan (3). Most media are based on the ability of *leuconostocs* to utilize citrate, which is recognized by the presence of halos around colonies growing on media containing insoluble calcium citrate (3, 7, 20). Such differentiation is not accurate since not all *leuconostocs* utilize citrate; furthermore, other bacteria associated with green plants, such as *Lactobacillus* species (12) and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, utilize citrate (19, 20). Vancomycin resistance in *Leuconostoc* species (6, 17, 21), as well as the sensitivity of *Lactococcus* species (6, 17) and some *lactobacilli* (21) to this antibiotic, led us to consider it for use in a *Leuconostoc* selective medium (LUSM medium). In combination with other ingredients these agents proved to be successful. In this paper we describe the use of LUSM medium for isolating *Leuconostoc* species from several natural sources and food products.

The microorganisms used in this study included bacteria and yeasts (Table 1). These organisms were stored in litmus milk at -20°C. Working cultures of lactococci were propagated in M17G medium containing 0.5% glucose (22); other organisms were propagated in MRS broth (4) by using 1%

inocula and overnight incubation at 30°C. The yeasts were maintained on slants of nutrient agar (Biokar) at 4°C and were propagated in nutrient broth (Biokar) incubated overnight at 25°C.

The basal medium contained 1.0% glucose, 1.0% Bacto Peptone (Difco), 0.5% yeast extract (BBL), 0.5% meat extract (Difco), 0.25% gelatin (Difco), 0.5% calcium lactate, 0.05% sorbic acid, 75 ppm of sodium azide (Sigma), 0.25% sodium acetate, 0.1% (vol/vol) Tween 80, and 15% tomato juice. Fresh tomatoes were blended in water (1:1, wt/vol) by using a Moulinex blender. The juice was then centrifuged at 8,000 × g in a Sorvall centrifuge for 15 min and used directly without filtration. The basal medium was sterilized by autoclaving it at 121°C for 15 min. The final pH of the medium varied from 5.3 to 5.8. If the medium pH was below 5.0, it was adjusted with NaOH to pH 5.5. Stock solutions of vancomycin (Sigma), tetracycline (Serva), and cysteine hydrochloride (BDH) were prepared as described below. Vancomycin and cysteine hydrochloride were dissolved in water to final concentrations of 10 mg/ml and 1 g/ml, respectively. A tetracycline solution was prepared as described by Maniatis et al. (14) by dissolving tetracycline in ethanol to a final concentration of 5 mg/ml. All of these solutions were filter sterilized by using 0.22-µm-pore-size Millipore membrane filters, and aliquots were stored at -20°C. The tetracycline solution was protected from light by covering the container with aluminum foil.

To prepare the complete medium, the basal medium was melted and tempered to about 48°C. The vancomycin, tetracycline, and cysteine hydrochloride solutions then were added to final concentrations of 30 and 20 µg/ml and 0.5 mg/ml, respectively.

Nine strains were tested for resistance to vancomycin (Table 1). Vancomycin was added to MRS agar at concentrations of 30, 100, 300, and 500 µg/ml, and the agar preparations were poured into sterile petri dishes and al-

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TABLE 1. Bacteria and yeasts used in this study, their origins, and their sensitivity to vancomycin

Organism	Source ^a	MIC of vancomycin (μg/ml)
Bacteria		
<i>Lactococcus cremoris</i> ACI	FDRC	<30
<i>Lactococcus diacetylactis</i> F7/22	FDRC	<30
<i>Leuconostoc cremoris</i> 225	OSU	>500
<i>Lactococcus lactis</i> 7962	OSU	
<i>Lactococcus lactis</i> LB11	IAV	
<i>Leuconostoc cremoris</i> 44-4		>500
<i>Leuconostoc cremoris</i> CAF-7	OSU	>500
<i>Leuconostoc cremoris</i> 104	OSU	>500
<i>Lactococcus diacetylactis</i> BU2	FDRC	<30
<i>Escherichia coli</i> V517	OSU	
<i>Micrococcus flavus</i>	OSU	
<i>Staphylococcus aureus</i>	IAV	
<i>Leuconostoc cremoris</i> M71	FDRC	>500
<i>Lactobacillus bulgaricus</i>	Yogurt starter (Rediset)	
<i>Streptococcus thermophilus</i>	Yogurt starter (Rediset)	
Yeasts		
<i>Saccharomyces cerevisiae</i>	IAV	
<i>Candida milleri</i>	IAV	

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lowed to harden and dry. One drop (25 μl) of an overnight culture of each test microorganism was spread onto the surface of an MRS agar plate containing each concentration of vancomycin. The plates were then incubated at 30°C and checked for growth after 2 and 4 days.

All of the organisms used in this study (Table 1) were propagated either in M17G medium (bacteria) or in nutrient broth (yeasts). To determine the ability of each organism to grow on LUSM medium, one 25-μl drop from an overnight culture (15 to 18 h) was spread onto the surface of the medium, and the preparation was incubated at 30°C and examined daily for growth for up to 4 days.

A total of 116 microorganisms were isolated from different products, including vegetables, milk, and curdled milk. Samples (11 g) of vegetables were blended with 99-ml portions of sterile distilled water for 30 s, serial dilutions were plated onto LUSM medium, and the preparations were incubated at 30°C for 72 h. Serial dilutions of blended vegetables and milk products were made in sterile 0.1% peptone water. Colonies were selected from diverse locations on plates containing uncrowded colonies and were purified on MRS agar. They were then stored in litmus milk at -20°C until they were needed. Isolates were identified by examining the following four *Leuconostoc* characteristics: morphology, catalase production, gas production from glucose, and arginine hydrolysis. Strains were identified to species level as described by Garvie (9, 10). The ability of the isolates to ferment the following sugars was also examined: lactose, sucrose, cellulose, arabinose, galactose, fructose, xylulose, trehalose, maltose, glucose, and esculin.

Comparisons of growth on MRS medium and LUSM medium were performed with two strains, *Leuconostoc cremoris* JLL8, and *Leuconostoc dextranicum* 181. These strains were grown separately in MRS broth at 30°C for 16 to 18 h. The cultures were then serially diluted, and colonies were counted by plating the organisms onto the two media.

Five replications were done with *Leuconostoc cremoris* JLL8, and three replications were done with *Leuconostoc dextranicum* 181, each in duplicate. The Student *t* test was used to perform a statistical analysis of the data.

Plasmid analyses were carried out by using the procedure of Anderson and McKay (1), as modified by Wyckoff et al. (24).

Our results are shown in Table 1. All *Lactococcus* strains were sensitive to vancomycin at concentrations less than 30 μg/ml, while all *Leuconostoc* strains were sensitive to vancomycin at concentrations less than 30 μg/ml, while all *Leuconostoc* strains were resistant to this antibiotic at concentrations greater than 500 μg/ml. The resistance of leuconostocs to vancomycin is well established (6, 11, 17, 21). Orberg and Sandine (17) and Simpson et al. (21) found that members of this genus are resistant to more than 2 mg of vancomycin per ml. Of 17 strains of *Leuconostoc* spp. studied by Orberg and Sandine (17), 3 without plasmids also could withstand up to 2 mg of this drug per ml. Lactobacilli (6, 21) and pediococci (21) also have been shown to be resistant to this antibiotic, while *Lactobacillus bulgaricus* tolerates concentrations only up to 100 μg/ml.

Lactococcus spp., *Lactobacillus bulgaricus*, and the yeasts which we tested were all inhibited on LUSM. Although *Lactobacillus bulgaricus* grew on MRS medium containing vancomycin, it did not grow on LUSM medium, suggesting that other ingredients of the medium were inhibitory for this organism.

With one exception, all of the isolates were gram-positive, catalase-negative, gas-producing cocci which did not hydrolyze arginine and were considered leuconostocs. The exception did not produce gas and formed cocci assembled in tetrads, characteristics that are typical of pediococci. A total of 89 *Leuconostoc* strains were identified to the species level. Our results showed that the most commonly isolated *Leuconostoc* species was *Leuconostoc oenos* (40.4% of the isolates), which may have been due to stimulation of this organism by cysteine hydrochloride (9). Other *Leuconostoc* species were isolated at the following frequencies: *Leuconostoc cremoris*, 13.5%; *Leuconostoc dextranicum*, 11.2%; *Leuconostoc mesenteroides* subsp. *mesenteroides*, 7.9%; *Leuconostoc mesenteroides* subsp. *paramesenteroides*, 16.9%; and *Leuconostoc lactis*, 10.1%. No lactobacilli were found among the isolates despite the facts that many lactobacilli are resistant to vancomycin and that lactobacilli have the same natural habitat as leuconostocs (6, 8). In isolating *Leuconostoc* from nature or food samples, the combination of the presence of agents inhibitory to most lactic acid bacteria other than *Leuconostoc* spp. and the presence of agents stimulatory for *Leuconostoc* spp. in LUSM medium selects for *Leuconostoc* spp. Sodium azide has been reported to be inhibitory for most lactic acid bacteria, including lactobacilli (13), but not for leuconostocs (15) or *Lactococcus diacetylactis* (20a). Therefore, this compound was used by Mayeux et al. (15) as a basis for the formulation of a *Leuconostoc* selective medium. Tetracycline has also been shown to be inhibitory for lactococci at concentrations less than 10 μg/ml (16). To our knowledge, no data are available on the effect of this antibiotic on lactobacilli or pediococci; nonetheless, tetracycline has been used in media that are selective for leuconostocs (16, 18).

The major contribution of vancomycin in our medium is to inhibit lactococci and homofermentative lactobacilli (21). The latter group also is inhibited by sorbic acid, and the sensitivity of these organisms to sorbate increases at pH values below 6.3 (21). In contrast, sorbic acid was shown to

stimulate the growth of leuconostocs (5); it is also inhibitory for yeasts (2).

No significant difference ($P < 0.05$) was found between the *Leuconostoc* counts on MRS medium and LUSM medium. For strain JLL8 the values obtained on MRS medium and on LUSM medium were $2.0 \times 10^8 \pm 1.7 \times 10^8$ and $1.8 \times 10^8 \pm 1.3 \times 10^8$ CFU/ml, respectively (means \pm standard deviations of five determinations performed in duplicate). The values obtained for strain 181 on MRS medium and on LUSM medium were $1.9 \times 10^8 \pm 2.7 \times 10^7$ and $1.6 \times 10^8 \pm 3.4 \times 10^7$ CFU/ml, respectively (means \pm standard deviations of three determinations performed in duplicate). For *Leuconostoc dextranicum* 181, a suitable colony size on LUSM medium was obtained after 3 to 5 days of incubation, while on MRS medium only 24 h of incubation was required. In view of these results, LUSM medium is recommended for the isolation and the enumeration of leuconostocs in mixed starter cultures or in fermented products containing a heterogeneous flora.

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REFERENCES

- Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Microbiol.* **46**:549-552.
- Beuchat, L. R. 1981. Synergistic effects of potassium sorbate and sodium benzoate on thermal inactivation of yeasts. *J. Food Sci.* **46**:771-777.
- Cogan, M. T. 1985. The leuconostocs: milk products, p. 25-40. In S. E. Gilliland (ed.), *Bacterial starter cultures for foods*. CRC Press, Boca Raton, Fla.
- de Man, J., M. Rogosa, and M. E. Scharpe. 1972. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23**:130-135.
- Emard, L. O., and R. H. Vaughn. 1952. Selectivity of sorbic acid media for the catalase-negative lactic acid bacteria and clostridia. *J. Bacteriol.* **62**:599-603.
- Facklam, R. D., J. G. Hollis, and M. D. Collins. 1989. Identification of gram-positive coccal and coccobacillary vancomycin-resistant bacteria. *J. Clin. Microbiol.* **27**:724-730.
- Galesloot, T. E., F. Hassing, and J. Stadhouders. 1964. Agar media voor het isoleren entellen van aromabacterian in zuursels. *Neth. Milk Dairy J.* **15**:127-150.
- Garvie, E. I. 1967. *Leuconostoc oenos* sp. nov. *J. Gen. Microbiol.* **48**:431-438.
- Garvie, E. I. 1984. Separation of species of the genus *Leuconostoc* and differentiation of the leuconostocs from other lactic acid bacteria. *Methods Microbiol.* **16**:147-178.
- Garvie, E. I. 1986. Genus *Leuconostoc*, p. 1071-1075. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore.
- Horowitz, H. E., S. Handwerger, K. G. van Horn, and G. P. Worsmer. 1987. *Leuconostoc*, an emerging pathogen. *Lancet*. **ii**:1329-1330.
- Keenan, T. W., and R. C. Lindsay. 1968. Diacetyl production and utilization by *Lactobacillus* species. *J. Dairy Sci.* **51**:188-191.
- Kenner, B. A., H. F. Clark, and D. S. Sarawat. 1966. Fecal streptococci. I. Cultivation and enumeration of streptococci in surface waters. *Appl. Microbiol.* **9**:15-20.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*, 8th ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mayeux, J. V., W. E. Sandine, and P. R. Elliker. 1962. A selective medium for detecting *Leuconostoc* organisms in mixed-strain starter cultures. *J. Dairy Sci.* **45**:655.
- McDonough, R. F., R. E. Hargrove, and R. P. Tittsler. 1963. Selective plating medium for *Leuconostoc* in mixed lactic starters. *J. Dairy Sci.* **46**:386-390.
- Orberg, P. K., and W. E. Sandine. 1984. Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc* spp. *Appl. Environ. Microbiol.* **48**:1129-1133.
- Pearce, L. E., and A. C. Halligan. 1978. Cultural characteristics of *Leuconostoc* strains from cheese starters, p. 520-521. In 20th International Dairy Congress. Congrailait, Paris.
- Reddy, M. S., E. R. Vedamuthu, and G. W. Reinbold. 1970. A differential broth for separating the lactic streptococci. *J. Milk Food Technol.* **34**:43-45.
- Reddy, M. S., E. R. Vedamuthu, J. Washam, and G. W. Reinbold. 1972. Agar medium for differential enumeration of lactic streptococci. *Appl. Microbiol.* **24**:947-952.
- 20a. Sandine, W. E. Unpublished data.
- Simpson, J. W., J. R. M. Hammond, and R. B. Miller. 1988. Avopracin and vancomycin, useful antibiotics for the isolation of brewery lactic acid bacteria. *J. Appl. Bacteriol.* **64**:299-309.
- Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29**:807-813.
- Teuber, M., and A. Geis. 1981. The family *Streptococcaceae* (nonmedical aspects), p. 1614-1630. In M. P. Starr (ed.), *The prokaryotes*, vol. 2. Springer-Verlag, New York.
- Wyckoff, A. H., W. E. Sandine, and J. K. Kondo. 1991. Transformation of dairy *Leuconostoc* using plasmid vectors from *Bacillus*, *Escherichia*, and *Lactococcus* hosts. *J. Dairy Sci.* **74**:1454-1460.